BRILLIANT GREEN AS A BACTERICIDAL AGENT FOR THE PURIFICATION OF VACCINE VIRUS

CHARLES KRUMWIEDE, JR., FRANK S. FIELDER,
AND
THOMAS A. WATSON

From the Bureau of Laboratories, Department of Health, New York City

As far as we know Churchman[1] is the only investigator who has made observations on the effect of strongly bactericidal anilin dyes on vaccine virus. He gives no details stating simply that the potency of vaccine virus was unimpaired by staining with gentian violet.

We were led to investigate the influence of anilin dyes on the potency and bacterial content of vaccine virus because of the frequent presence of gas-producing anaerobic bacilli giving positive reactions with the official hygienic laboratory tests for the presence of B. welchii; and because of the continued viability of these bacilli in spite of the glycerole and carbolic acid added to purify and preserve the virus.

Brilliant green was used because of its wider range of bactericidal action[2] as compared with the violet dyes and especially because of its action on bacilli of the colon group.

The data here presented in this article show that brilliant green has little, if any, influence on the potency of vaccine virus, and added to the present glycerol-carbolic preservative, gives a more efficient and more rapidly bactericidal preservative.

The accompanying tables give the bactericidal results obtained with virus artificially inoculated with the bacterial types considered most objectionable in virus.

Brilliant green, therefore, added to heavily inoculated glycerol-carbolic virus led to a rapid reduction of the inoculated bacteria and sterilization in 6 days, whereas with glycerol-carbolic alone the reduction was slower and sterilization was not complete in 6 days.

In this connection it is well to note the persistence of this type when encountered in glycerol-carbolic virus as prepared for distribution.

Received for publication October 9, 1917.

[1] Proc. Soc. Exper. Biol. and Med., 1914, 11, 55.
[2] Krumwiede, C., and Pratt, J. S.: Jour. Exper. Med., 1914, 19, 501.

TABLE 1

VACCINE VIRUS TO WHICH WAS ADDED ONE-FOURTH OF ITS VOLUME OF A HEAVY MILKY SUSPENSION OF STAPHYLOCOCCI AND STREPTOCOCCI. KEPT IN REFRIGERATOR BETWEEN TESTS

Dates Tested 0.1 C.c. Plated, Blood-agar	Inoculated Glycerol-Carbolic Virus Containing Brilliant Green; Concentration, 1:10,000	Inoculated Glycerol-Carbolic Virus; Saline to Same Volume as Preceding
July 22, 1915.....	Plates, solid very closely crowded fine colonies	Plates as preceding
July 23.....	33% reduction in number of colonies	No evident reduction
July 24.....	40% reduction	5% reduction
July 26.....	99% reduction	15% reduction
July 27.....	Three colonies total on 3 plates	50% reduction
July 28.....	No growth	98% reduction

TABLE 2

VIRUS CONTAINING LARGE NUMBERS OF BACILLI GIVING POSITIVE B. WELCHII TESTS WAS FURTHER INOCULATED WITH CULTURES OF A SIMILAR BACILLUS ISOLATED FROM PREVIOUS VIRUS. THE VIRUS WAS THE ROUTINE GLYCEROLE-CARBOLIC SUSPENSION

Glucose-Fermentation Tubes Inoculated	Inoculated Virus	Inoculated Virus, Brilliant Green 1:5000	Inoculated Virus, Brilliant Green 1:10,000
At time of mixing	90% gas	No gas; probably dye inhibition	100% gas
After 2 days	95% gas	No gas	No gas
After 6 days	60% gas	No gas	No gas

TABLE 3

VIRUS HEAVILY INOCULATED WITH TETANUS SPORES OBTAINED FROM BROTH CULTURE SEDIMENT

Tested	Inoculated Virus	Inoculated Virus, Brilliant Green 1:5000	Inoculated Virus, Brilliant Green 1:10,000
At once	Tetanus bacilli isolated	Tetanus bacilli isolated	Tetanus bacilli isolated
After 248 days	Tetanus bacilli isolated	Tetanus bacilli isolated	Tetanus bacilli isolated

Virus 2260, collected April 14, 1915; B. welchii types still present, March 1, 1916.

Virus 2261, collected April 28, 1915; B. welchii types found May 6, 1916, not found June 9, 1916.

Virus 2262, collected May 12, 1915; B. welchii types found May 16, not found June 9, 1916.

Virus 2263, collected May 12, 1915; B. welchii types found June 11, 1916.

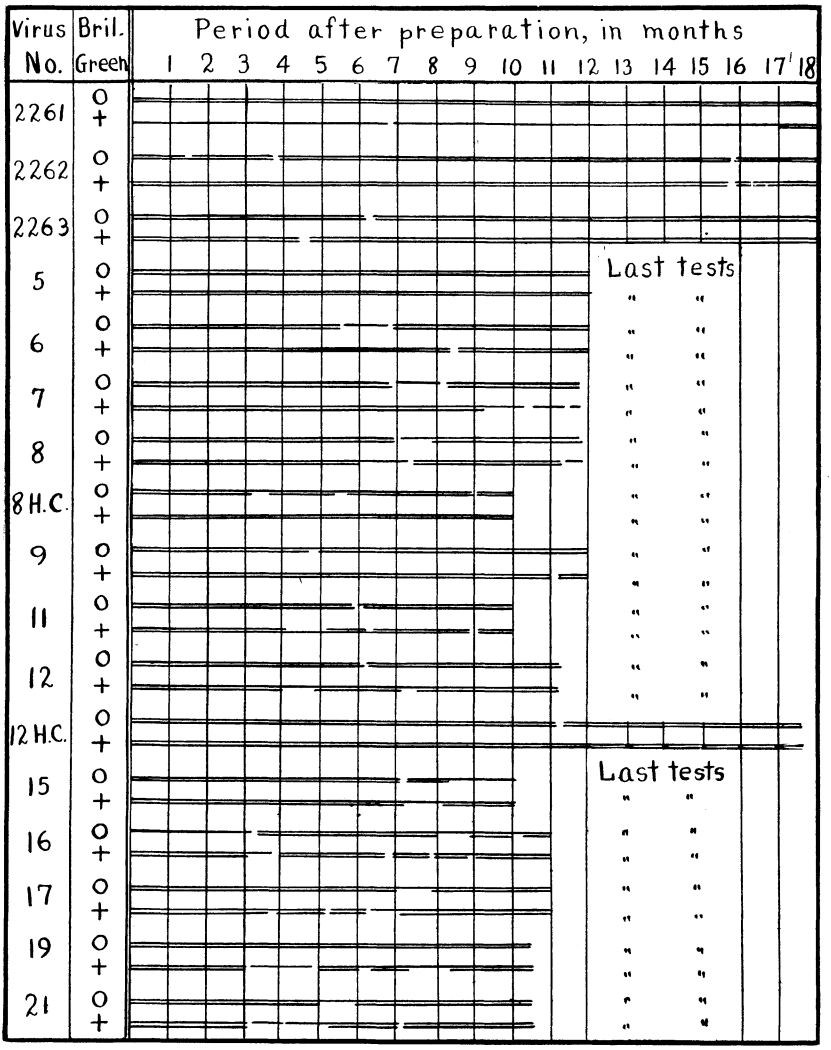
No quantitative estimations were made, but based on the amount of growth obtained in the primary "shake" cultures made from the virus, numerous spores were present at the end of the experiment.

Although tetanus spores are evidently highly resistant, this is indirectly an advantage over the present preservative. If tetanus bacilli were present, they should be easily recovered due to the killing of the associated bacteria and spores, especially of B. welchii types which otherwise by overgrowth nullify the accepted tests for the presence of tetanus bacilli or spores.

Before instituting a series of tests of viruses with and without brilliant green, it seemed well to determine whether the carbolic acid was of added value when the dye was employed. With the carbolic acid, the disappearance of some undetermined types of cocci was more rapid. Comparative tests also revealed that a concentration of at least 1 : 10,000 of the dye was necessary to obtain bacteriologic sterility in a relatively short time. A greater concentration, 1 : 5000, was not appreciably more effective, and as at this concentration there was some indication of injury to the vaccine virus itself, practically all the subsequent observations were made with a concentration of 1 : 10,000.

Evidently brilliant green when added to glycerol-carbolic virus markedly hastened its purification. As frequently the first test on the brilliant green virus gave negative cultures the actual earliest day on which a bacteriologically sterile preparation was obtained cannot be stated. Because of this, the following comparison of averages is distinctly in favor not of the virus thus treated, but in favor of the glycerol-carbolic virus. Thus, with the brilliant green virus the average day on which gas was not noted was 11 days; growth not noted 24 days; with the glycerol-carbolic virus, gas was still positive after an average of 33 days, and growth was still positive after an average of 119 days.

The accompnying chart gives a summary of the potency tests of vaccine virus with and without brilliant green. The concentration of brilliant green employed was 1 : 10,000.



Duration of potency of vaccine virus with and without brilliant green.

Double line=100% positive takes of highest quality. Single line=100% positive takes of moderate quality. Break in both lines=less than 100% takes. Break in one line=break in quality of takes. "Takes"=primary vaccinations of children. (We are indebted to Drs. Samuel Parnass and Julius Blum who carried out the clinical tests on children). +=brilliant green added. O=no brilliant green, glycerol-carbolic virus.

TABLE 4

COMPARATIVE RATE OF PURIFICATION OF GLYCEROL-CARBOLIC VIRUS WITH AND WITHOUT BRILLIANT GREEN. TESTED BY INOCULATION OF GLUCOSE BROTH IN FERMENTATION TUBES. DAY NOTED OF LAST POSITIVE AND FIRST NEGATIVE TEST FOR GAS PRODUCTION; OF LAST DAY OF CLOUDING AND FIRST DAY OF ABSENCE OF GROWTH. TESTED ABOUT EVERY 7 TO 14 DAYS. GAS NOTED AS ITS DEVELOPMENT IS AN INDICATION OF THE PRESENCE OF B. WELCHII TYPES

Virus Number	Brilliant Green 1:10,000				Brilliant Green not Added			
	Gas	Days	Growth	Days	Gas	Days	Growth	Days
14	+ —	2 3	+ —	11 20	+ —	30 39	+ ——	92 ——
15	+ —	10 21	+ —	82* 91	+ ——	93 ——	+ ——	266 ——
16	—— —	—— 9**	—— —	—— 9**	+ ——	43 ——	+ ——	45 ——
17	—— —	—— 9**	—— —	—— 9**	+ —	4 17	+ ——	45 ——
19	—— —	—— 16**	—— —	—— 16**	+ ——	44 ——	+ ——	44 ——
21	+ —	1 8	+ —	24 29	+ —	32 42	+ ——	199 ——
22	+ —	3 10	+ —	26 32	+ —	14 21	+ ——	193 ——
23	—— —	—— 11**	—— —	—— 11**	+ —	13 31	+ —	156 171
24	—— —	—— 12**	—— —	—— 12**	+ —	4 13	+ ——	147 ——
25	—— —	—— 9	—— —	—— 9	+ —	71 94	+ —	71 94
26	—— —	—— 13	—— —	—— 13	+ —	28 55	+ ——	112 ——
27	—— —	—— 13	+ —	28 37	+ —	14 21	+ —	57 71

* Only organism after 2 months, small coccus. ** No previous tests.

Although variations in the degree of "takes" are sometimes in favor of the virus, without brilliant green the reverse also occurs. On the whole, there is no evidence of the dye exerting any appreciable deleterious influence, especially if the duration of potency is considered. Probably some of the variations in results are dependent on inherent variables in the method of testing.

As a control on the foregoing human vaccinations, vaccinations were carried out on rabbits with dilutions of treated and untreated vaccine, as explained in Table 5.

The results recorded in Table 5 show no influence of the dye on the total content of virus after exposure for 40 days.

We have employed brilliant green to preserve the seed-virus collected from calves vaccinated with human virus as well as to preserve

TABLE 5

POTENCY TESTS ON RABBITS VACCINATED WITH VACCINE VIRUS WITH AND WITHOUT BRILLIANT GREEN DYE 1:10,000. VIRUSES 5 AND 6 WERE EXPOSED TO BRILLIANT GREEN, MARCH 31, 1916

Virus Number and Date of Test	Without Brilliant Green — Dilution of Virus	Results	With Brilliant Green 1:10,000 — Dilution of Virus	Results
June 9, 1916				
Virus 5	Undiluted	Good confluent take	Undiluted	Good confluent take
Virus 5	Diluted 1:10	Good confluent take	Diluted 1:10	Good confluent take
Virus 5	Diluted 1:100	Good confluent take	Diluted 1:100	Good confluent take
Virus 5	Diluted 1:1000	Good confluent take	Diluted 1:1000	Good confluent take
June 9, 1916				
Virus 6	Undiluted	Good confluent take	Undiluted	Good confluent take
Virus 6	Diluted 1:10	Good confluent take	Diluted 1:10	Good confluent take
Virus 6	Diluted 1:100	Good confluent take	Diluted 1:100	Good confluent take
Virus 6	Diluted 1:1000	Good confluent take	Diluted 1:1000	Good confluent take

the seed-virus from rabbits vaccinated with this human-calf virus. This rabbit virus is used to vaccinate calves in order to obtain virus for general distribution; the interpolation of the rabbit avoiding calf to calf inoculation which commonly has a deleterious effect on its potency.

In Table 6 the seed exposed and the time of exposure are given.

The addition of brilliant green to the seed virus had no effect on the potency of the final virus. In no instance was any appreciable diminution noted in the intensity of the "take" nor in the amount of yield of virus from the animals vaccinated.

At first the vaccine virus was treated with brilliant green by adding 1 part of a 1:1000 solution of the dye to 9 parts of the finished

TABLE 6

TYPE OF SEED EXPOSED, TIME OF EXPOSURE AND RESULTS

Human-Calf Virus; Number and Date of Exposure	Rabbit Seed; Number and Date of Exposure	Calf Vaccine; Number and Date Vaccinated	Duration of Potency of Calf Vaccine in Days
8 June 10, 1916	1 July 18, 1916	18 Aug. 9, 1916	Still good at 300 days
	2 July 25, 1916	19 Aug. 24, 1916	Still good at 294 days
		22 Sept. 29, 1916	Still good at 301 days
12 June 10, 1916	1 Oct. 9, 1916	24 Nov. 17, 1916	Still good at 253 days
		25 Dec. 7, 1916	Still good at 233 days
	3 Nov. 8, 1916	27 Dec. 21, 1916	Still good at 218 days
		28 Jan. 23, 1917	Still good at 175 days
	4 Jan. 16, 1917	32 Feb. 8, 1917	Still good at 168 days
	5 Feb. 5, 1917	33 Feb. 21, 1917	Still good at 155 days

Explanation: Numbers refer to animal collections. From left to right gives passages of each collection from calf to rabbit to calf.

glycerol-carbolic virus. Later the pulp from the calf was ground up in a glycerol-carbolic solution containing 10% less water and this volume of brilliant green solution (1 : 1000) added, thus avoiding any dilution of the finished product as compared with the routine glycerol-carbolic virus.

In observing the results of vaccinations made with the brilliant green-treated virus, there has been some indication that the secondary inflammation was reduced. Possibly this effect could be enhanced by subsequent applications of the dye-solution to vaccination, limiting in this way the multiplication of the skin cocci.

The sterilization of the virus by brilliant green may find an important practical application in that intermediate sterilization would allow calf to calf vaccination, without loss in potency of the virus. Without bacterial sterilization such a procedure quickly leads both to degeneration of the virus and decreasing yields, due apparently to a great extent to the overgrowth of the calf bacterial flora enriched by the transfer of this flora from calf to calf. At present this overgrowth is avoided by obtaining the seed for calf vaccination from rabbits.

The use of brilliant green alone, although somewhat less efficacious than in combination with carbolic acid, may be of service in obtaining a bacteriologically sterile virus for attempts at cultivation. The observation of Churchman and Russell[3] that certain animal tissues grow readily in concentrations of gentian violet which are bactericidal is suggestive in this connection.

CONCLUSIONS

Brilliant green in a concentration of 1 : 10,000 has no appreciable effect on the potency of vaccine virus. This amount of dye when used in combination with the glycerol-carbolic solution usually employed markedly hastens the rate of reduction of the bacterial content, rendering most preparations bacterially sterile in from 2-4 weeks.

Although the dye cannot be relied on to kill tetanus spores should they be present, no difficulty should be encountered in demonstrating their presence after the associated bacteria are destroyed.

The results obtained warrant its practical application to vaccine virus for general distribution or at least in emergencies when the virus must be employed shortly after collection from the calf.

The use of brilliant green gives a simple method hitherto not available for obtaining a bacteriologically sterile but fully potent virus for experimental purposes.

[3] Proc. Soc. Exper. Biol. and Med., 1914, 11, 123.